The Actin and Myosin Filaments of Human and Bovine Blood Platelets

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Research Article

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ABSTRACT The contractility of platelets has been attributed to an actomyosin-like protein which has been well defined on a physicochemical basis. Moreover, platelets contain ±80 A filaments which resemble actin filaments in smooth muscle. Studies were undertaken on human and bovine platelets to better define the morphologic structures which may subserve this contractile function. In order to identify actin, the ability of the filaments to react with heavy meromyosin (HMM) was tested. Accordingly, platelets were glycerinated and treated with HMM. In addition, platelet actin was extracted, reacted with HMM, and examined by negative staining. In both instances typical arrowhead structures with clearly defined polarity and a periodicity of ±360 A formed. As is the case with purified muscle actin, the complexes were dissociable with Mg-ATP. The formation of myosin-like filaments was observed when osmotically shocked platelets were incubated with $MgCl_2$ and excess ATP. These "thick" filaments measured 250–300 A in width, tapered at both ends and often occurred in clumps. They resembled aggregates of thick filaments described in contracted smooth muscle. Extraction of platelets by methods suitable for the demonstration of myosin showed filaments with an average length of 0.3 μ, a smooth shaft, and frayed or bulbous ends. These appeared identical to those seen in synthetically prepared myosin of striated muscle. It is suggested that the filaments described here represent the actin and myosin of platelets.

INTRODUCTION

Platelets contain a contractile protein with the physicochemical properties of actomyosin (1–3). Ultrastructural studies of this material have shown microfilaments bearing a striking resemblance to muscle actin (4). However, since purification has remained incomplete, it has not been possible to conclude with certainty that these filaments represent the contractile protein. Identification of actin or myosin within intact platelets has also been fraught with difficulty. Although filaments are readily resolved in platelet pseudopods where these structures run parallel with the axis of the extended cellular process, the demonstration of similar filaments throughout the cytoplasm requires electron microscopy of platelets after their aggregation or other in vitro manipulation (5, 6). In general, the size and appearance of the cytoplasmic filaments seen under these conditions resemble the actin of smooth muscle (7, 8) or the "thin" filaments believed to represent this protein in skeletal muscle (9). Furthermore, the structures correspond to the actin filaments reported in many primitive motile organisms (10–12). On the other hand, characteristic "thick" filaments representing myosin in skeletal muscle have been less well defined in platelets. So far, the assumption that myosin is also present in platelets has rested on biochemical evidence primarily (13, 14). For these reasons, it seemed worthwhile to initiate studies designed to better delineate the morphologic components of the platelet contractile apparatus. In order to identify actin, use was made of the observation that heavy meromyosin (HMM[1]), the ATP-ase containing moiety of myosin (15) forms characteristic arrowhead complexes with purified muscle actin (9). Moreover, the delineation of actin filaments in situ has recently been facilitated by a method which permits entry of HMM into cells following their glycerination (16). Thus, the resolution of arrowhead complexes formed with isolated fibrils as well as those in sectioned embedded platelets could be achieved.

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[1] *Abbreviations used in this paper:* HMM, heavy meromyosin; LMM, light meromyosin; PRP, platelet rich plasma; SSS, standard salt solution.

Since no thick or myosin-like filaments had ever been described in platelets, a histochemical method which had been employed to localize myosin ATP-ase on thick filaments of skeletal muscle was attempted for this purpose (17). Under the conditions of this experiment, aggregates of thick filaments formed which closely resembled those seen in artificially prepared myosin as well as those recently described in contracted (18) or trypsin-treated smooth muscle (19). Similarly, thick filaments were found in platelet extracts prepared under conditions favorable for the demonstration of myosin.

These observations lend further support to the contention that platelets possess actin as well as myosin, and that the filaments to be depicted in this communication represent the morphologic counterparts of these proteins.

METHODS

Platelets. Human platelets were isolated from heparinized blood of normal volunteers essentially as described elsewhere (5). Anticoagulation of whole blood with trisodium citrate (3.8%) or a 10% solution of disodium ethylenediaminetetraacetate (EDTA), 0.01 ml/ml, did not alter the results. Bovine blood was collected in citrate during exsanguination by carotid section.[2] Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 2100 *g* for 8 min at room temperature. To prevent irreversible clumping of bovine platelets it was necessary to add 0.01 ml EDTA per ml PRP before centrifugation. Sedimentation of bovine platelets required 4000 *g* for 10 min at room temperature.

Actin. In order to demonstrate thin filaments and the formation of "arrowhead complexes" in situ platelets were glycerinated by a slight modification of the method described by Ishikawa (16). Platelets from 20–25 ml PRP were resuspended in 50% glycerol in a standard salt solution (SSS) containing 0.1 M KCl, 0.005 M $MgCl_2$, and 0.006 M phosphate buffer at pH 7.0 for 42–69 hr at 4°C. The cells were transferred to 25% glycerol for 5–6 hr and finally to 5% glycerol for 5–6 hr. From 0.02 to 0.05 ml of glycerinated platelets were resuspended in 1 ml HMM[3] and allowed to remain overnight at 4°C. The cells were then sedimented and fixed for electron microscopy as described below. The concentration of HMM ranged between 2 and 4 mg/ml in 0.5 M KCl, 10 mM Tris, 1.0 mM EDTA in 50% glycerol at pH 7.2.

For the purpose of negative staining, actin filaments were isolated from bovine platelets by a method used for the extraction of actin from Acanthamoeba castelanii (20). Twice the volume of 1.4 M KCl was added per volume of packed platelets. After thorough mixing by up and down pipetting the preparation was frozen and thawed twice and stored at −15°C until further use. Upon thawing, the pH of the solution was adjusted to 7.4, the preparation was then refrigerated for 1 hr, and subsequently centrifuged at 9000 *g* for 15 min at 4°C. The supernate was diluted 1:30 with 1.4 M KCl after which drops were placed on parlodion-carbon coated electron microscope grids for 5 min. The grids were drained with the tip of a piece of filter paper but not allowed to dry before the application of 1% uranyl acetate at pH 4.2 for the purpose of negative staining. To prepare arrowhead complexes, a drop of HMM (0.5 mg/ml) was placed directly on the grid holding the actin filaments. The grid was washed, and staining with uranyl acetate was carried out as described above.

Localization of ATP-ase. Platelets isolated from 20–30 ml human blood were placed in distilled water for 15 min at room temperature or at 37°C in order to preserve microtubules. The lysed cells were sedimented at 2500 *g* and resuspended in substrate according to the method of Tice and Smith (17). The incubation medium contained 0.002 M ATP, 0.05 M $MgCl_2$, and 0.005 M $PbNO_3$ at pH 6.5. The reaction was allowed to proceed with gentle agitation for 30 min at 37°C after which the platelets were sedimented and fixed in glutaraldehyde. Controls were prepared by omission of ATP or $PbNO_3$ from the incubation mixture, or by addition of 5×10^{-3} M mersalyl acid, a sulfhydryl inhibitor known to interfere with the action of muscle ATP-ase (21, 22).

Myosin. For the purpose of isolating myosin filaments from platelets the procedure of Kaminer was followed (23). To 1 ml packed bovine platelets, 10 ml 0.6 M KCl was added at pH 6.5. The cells were disrupted by freezing and thawing twice and kept overnight at 4°C. After centrifugation at 9000 *g*, the molarity of the KCl in the supernates was brought to 0.1 M by dilution with Tris-HCl buffer at pH 6.5. The preparation was agitated for 10 min at 4°C during the process of dilution. The supernate was placed on parlodion-carbon coated grids for 5 min which were then washed and stained with 1% uranyl acetate as described before.

Electron microscopy. The preparations destined for the study of filaments in situ were fixed in 3% glutaraldehyde (24) overnight and postfixed with 2% osmium tetroxide for 2 hr. After fixation, the specimens were washed and resuspended in 0.5% uranium acetate in saline for 1 hr. Dehydration and embedding in Epon 812 (Shell Chemical Co., New York) was accomplished by the procedure of Luft (25). Thin sections obtained with an LKB ultrotome (LKB Instruments, Inc., Rockville, Md.) were stained with uranyl acetate (26) and lead citrate (27). For negative staining, solutions containing filaments were placed on parlodion-carbon coated grids for 5 min without prior fixation. The grids were drained by blotting with the tip of a piece of filter paper but not allowed to dry before the application of 1% uranyl acetate at pH 4.2 for 5 min. A Siemens Elmiskop I electron microscope (Siemens Corp., Iselin, N. J.) equipped with a cooling device was used with accelerating voltage of 60 kv.

RESULTS

Actin filaments. When platelets were glycerinated their membrane permeability increased rapidly as swelling and rarefaction of the cytoplasm could be observed by phase microscopy within 1–2 min. On electron microscopy, glycerinated platelets revealed 70–80 A fila-

[2] We are indebted to Mrs. Jane Lenahan of Warner-Lambert Pharmaceutical Co., Morris Plains, N. J. for her kind help in arranging for the collections of bovine blood.

[3] HMM was kindly donated to us at various occasions by Dr. Carl Moos, Biophysics Department, State University of New York, Stony Brook, N. Y., Dr. David Woolley, Retina Foundation, Boston, Mass., and Dr. Evan Eisenberg, National Health and Lung Institute, National Institutes of Health, Bethesda, Md.

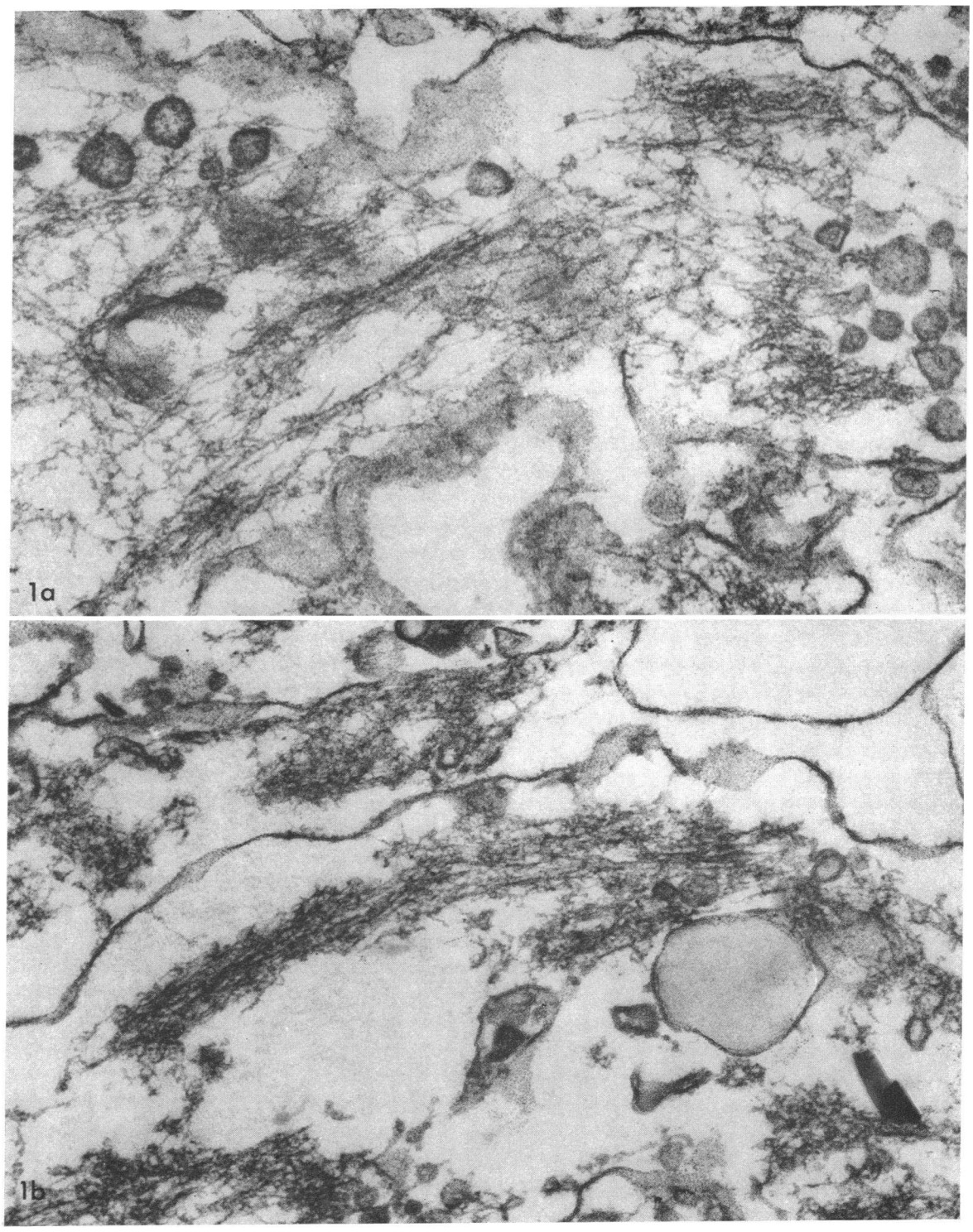
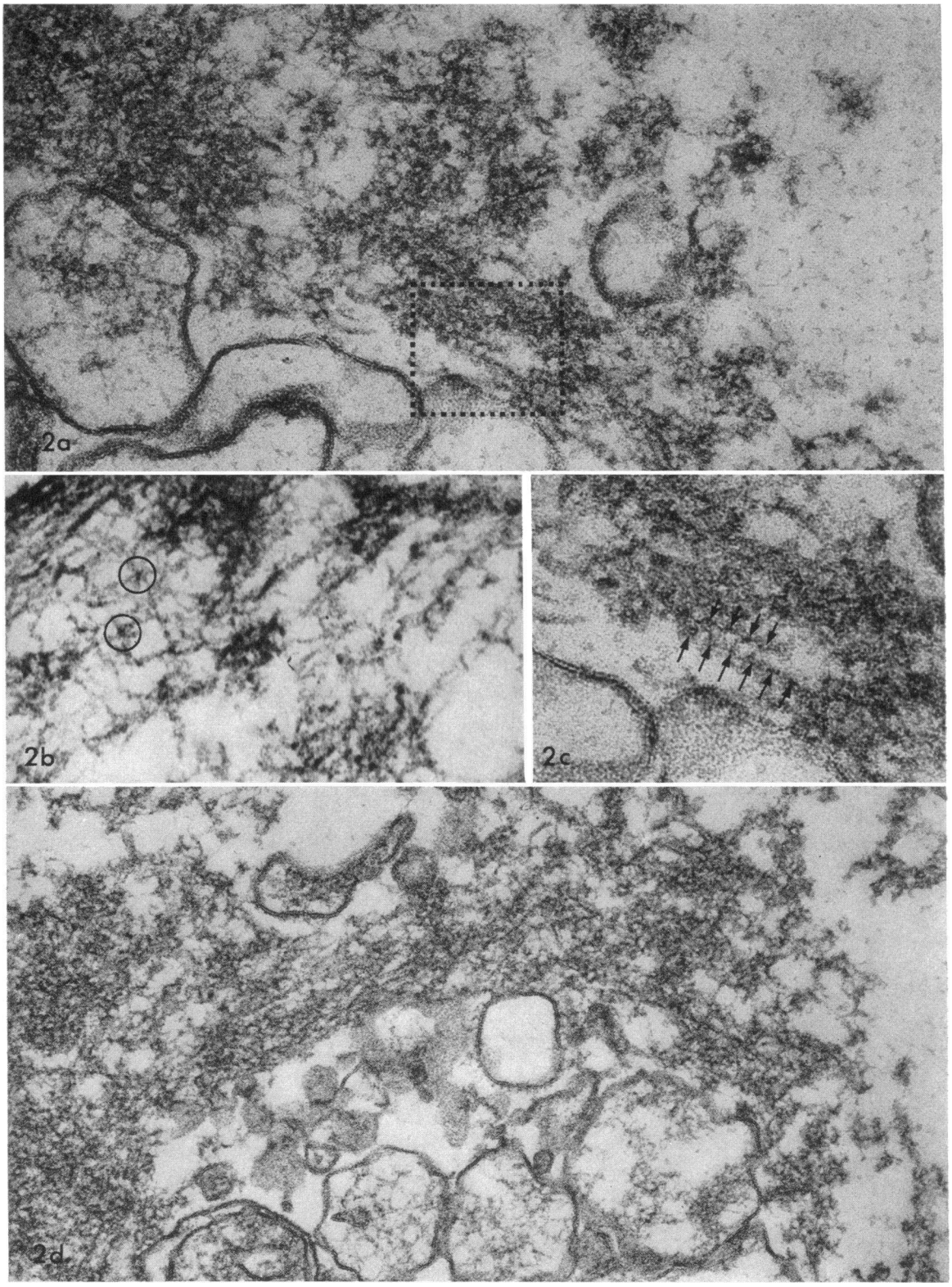


FIGURE 1 Details of platelets swollen by glyerination showing smooth randomly oriented 80 A filaments of indeterminate length. In Fig. 1b the filaments appear to form a bundle along the circumference of the platelet. × 75,000.


2a
2b
2c
2d

ments (Figs. 1a and b) which had been described previously after osmotic shock (5). When glycerinated platelets were treated with HMM, the filaments acquired a feathery appearance (Figs. 2a–d) resembling the "decorated" fibrils or "arrowhead complexes" described by Ishikawa in HMM-treated muscle and other tissue culture cells (16). Although a periodicity of 360 A could be made out (Fig. 2c) and an increase in width of the filaments was appreciated in longitudinal as well as in cross sections (Fig. 2b), the polarity of the projections on the filaments could not be clearly discerned in sectioned material. On the other hand, when isolated filaments were reacted with HMM the remarkable composite arrowhead structure was clearly defined (compare Figs. 3a and b with Figs. 4 and 5). The filaments had more than doubled in width and "arrowheads" all pointing in the same direction occurred with a periodicity of ±360 A along the length of each filament. The appearance of these structures was indistinguishable from those obtained when HMM is reacted with purified actin from striated or smooth muscle (See plates XIX and XX, Ref. 9). Moreover, the complex could be dissociated with 10^{-3} M ATP thus providing further evidence that the filaments represented actin. Arrowheads did not seem to form with microtubules or with the membranes of disrupted organelles. However, more extensive studies may be necessary to exclude the possibility that HMM reacts with the fibrils associated with the canaliculi or the surface membrane (28).

Myosin filaments. Unexpectedly, thick filaments were found in platelets which were treated with excess ATP and $MgCl_2$ for the histochemical demonstration of ATP-ase (Figs. 6–9). The distribution of the product of this reaction which could be detected when $PbNO_3$ was present in the substrate medium will not be illustrated here since it did not seem to be specific for the ATP-ase activity associated with the fibrils. However, the presence of mersalyl acid, an inhibitor of muscle ATP-ase (21, 22) prevented formation of any reaction product, though it did not interfere with the aggregation of thick filaments. In general, the filaments measured 250–350 A in diameter and up to 0.5 μ in length. They often occurred randomly oriented in clumps and had the shape of "tactoids" as they tapered at both ends (Fig. 8). Some of the filaments clearly consisted of laterally aggregated thinner filaments measuring about 20 A in width and fraying apart at both ends (Fig. 6, arrow). There was a striking resemblance of the aggregates described here to the myosin filaments reported recently in contracted (18) and trypsin-treated (19) smooth muscle. In rare cells, instead of the fairly uniform aggregates of thick filaments extremely large crystalline structures were observed. These measured 2–3 μ in length and up to 800 A in their largest diameter (Fig. 9). The fine 20–30 A filaments were also seen to compose these larger tactoids. In addition, cross striations with a periodicity of about 110 A were readily apparent. In general, the impression was gained that the platelets containing these large aggregates had a denser cytoplasm than those showing average sized thick filaments (Fig. 9).

Because of the greater availability of bovine platelets, they were used for the extraction and partial purification of myosin. On negative staining, the isolated myosin-like filaments had a fairly uniform length (Fig. 10). They presented a relatively smooth shaft which appeared to be composed of longitudinally arranged subunits and measured from 0.15–0.2 μ (Fig. 10 b–d). The tapered ends looked frayed or presented irregularly shaped projections at varying angles (Fig. 10e). At other times, the structure terminated in bulbous ends imparting a dumbell shape to the aggregates (10 a–d). Although there was some variation in thickness, the length of these filaments appeared fairly constant with an average of 0.3 μ when 50 filaments were measured. Thus, these structures are remarkably reminiscent of synthetically prepared myosin from striated (9) or smooth (23) vertebrate muscle. Illustrations of myosin isolated from the slime mold Physarum could virtually be superimposed on these plates (29).

DISCUSSION

Although contractile properties have been attributed to many vertebrate cells, apart from muscle, only platelets have so far been shown to contain an actomyosin-like protein. Moreover, it has been claimed on the basis of physicochemical studies that both actin and myosin can be distinguished in platelets (13) and that even a relaxing principle may play a role (30). With improvement in fixation, embedding, and other electron microscopic techniques thin fibrils resembling those in smooth

FIGURE 2 Details of glycerinated platelets which have been reacted with HMM. The filaments no longer appear "smooth" but are "feathery" or "decorated" when compared with those seen in Fig. 1. The filaments within the box in Fig. 2a are seen at higher magnification in Fig. 2c where the periodicity is indicated with arrows. In Fig. 2b the decorated filaments are seen in cross section (circles); the projections probably represent HMM. Fig. 2d shows a bundle of decorated filaments for comparison with the smooth bundle seen in Fig. 1b. Magnification: 2a, × 127,000; 2b, × 144,000; 2c, × 195,000; and 2d, × 90,000.

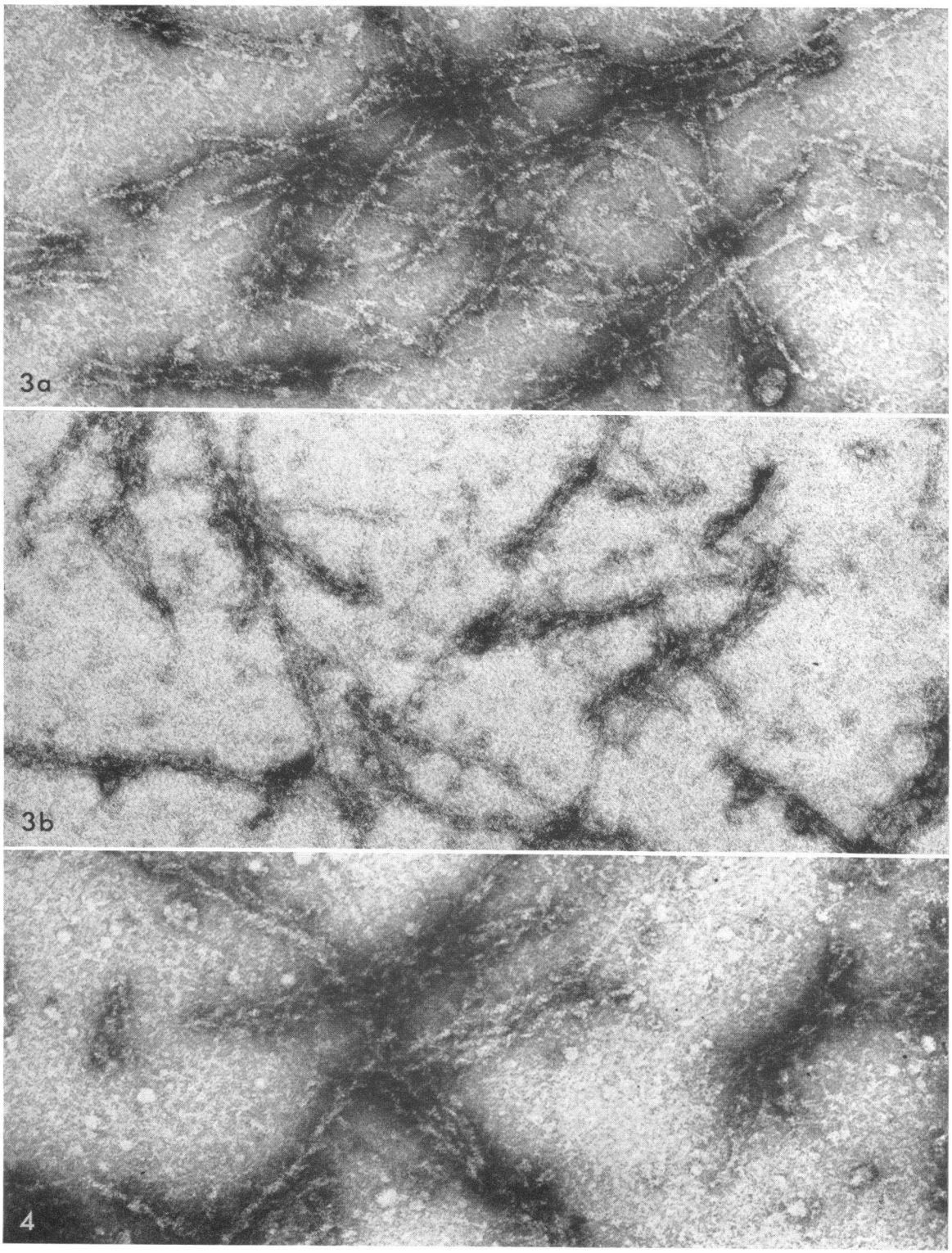


FIGURE 3 Thin filaments extracted from two specimens on different occasions. Although some impurities appear to be adherent, in general, the filaments are smooth when compared with Fig. 4 (bottom). × 120,000.

FIGURE 4 Thin filaments from specimen shown in Fig. 3b after reaction with HMM. The arrowhead complexes are evident. × 120,000.

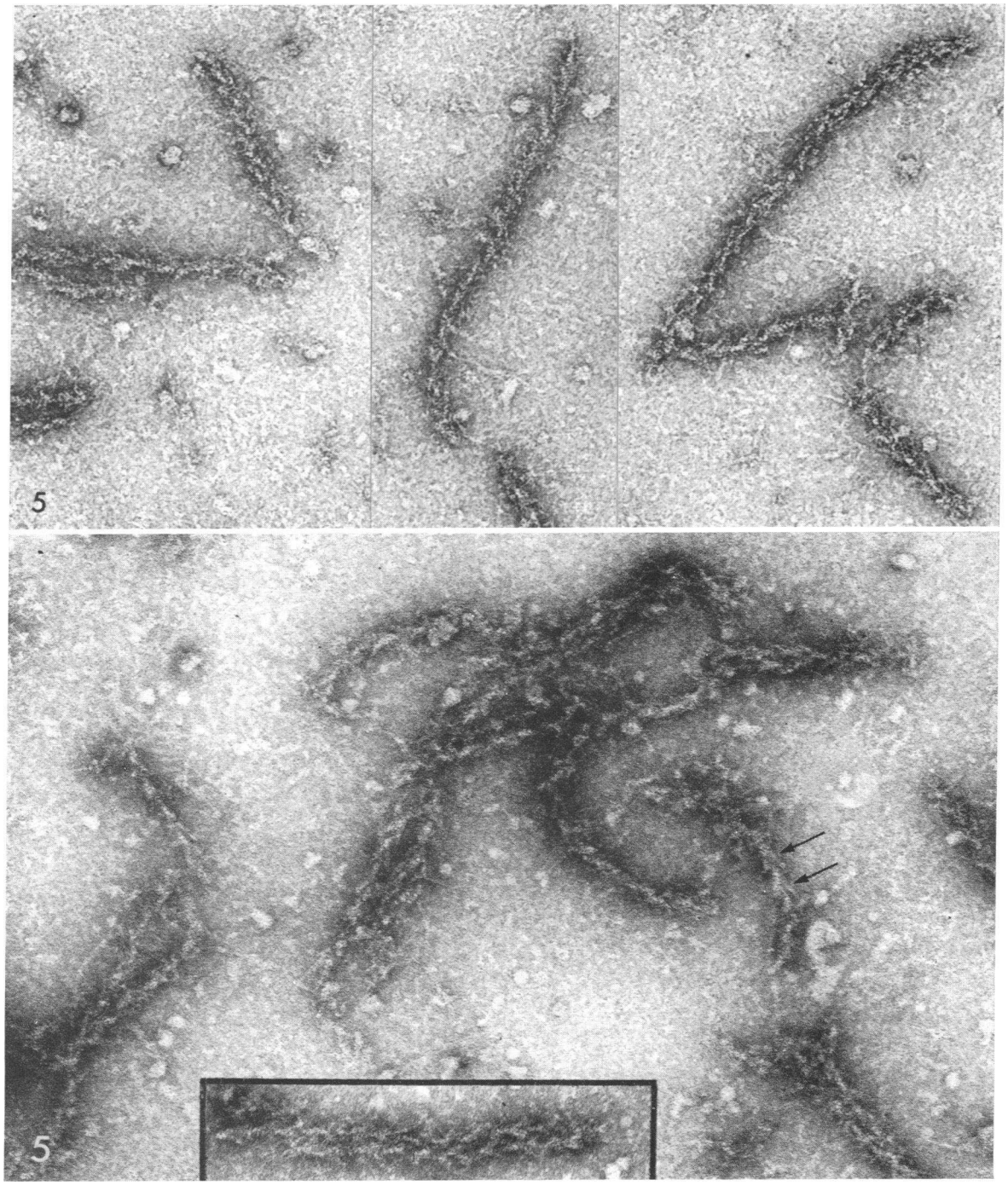
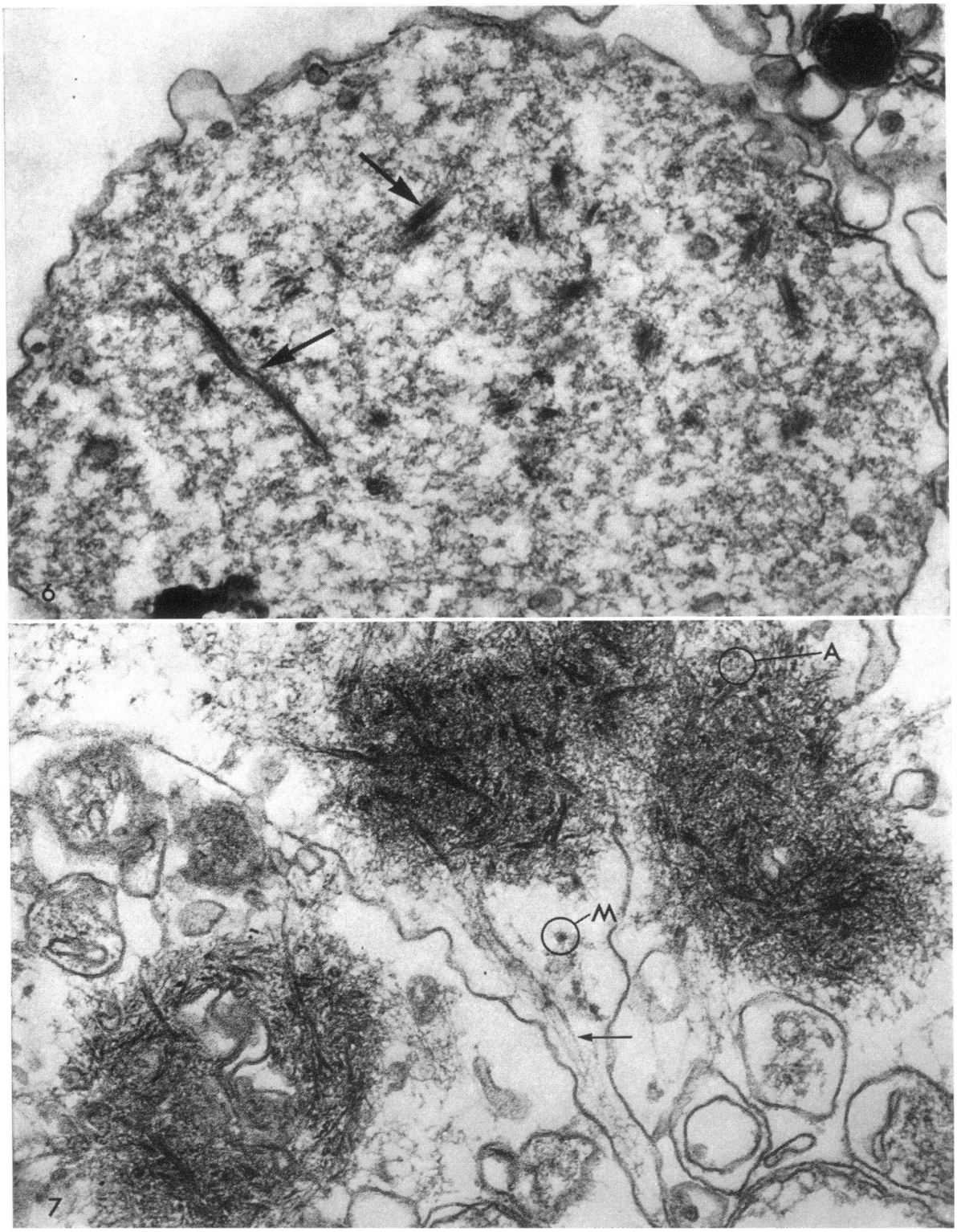


FIGURE 5 Thin filaments extracted on various occasions reacted with HMM and negatively stained. The characteristic polarity of the arrowhead arrays is clearly seen (arrows). Periodicity appears constant at ±360 A and all arrowheads on the same filament point in the same direction. Polarity shows particularly well on filaments in the inset. × 120,000.

muscle were also demonstrated in platelets. It remained necessary however to establish an identity between these fibrils and muscle actin and to adduce morphologic evidence for the existence of characteristic myosin. The first task seemed relatively simple since actin-like filaments were found in partially purified thrombosthenin, a protein which proved to have the ATP-ase activity, viscosity characteristics, and sedimentation properties



FIGURES 6 and 7 Details of platelets after osmotic shock incubated at 37°C in excess ATP, Ca^{++}, Mg^{++}. The aggregates seen in Fig. 6 clearly show lateral aggregations of 20 A filaments (arrows). × 54,000. Fig. 7 illustrates three clumps of filaments. Cross sections of thin, actin-like filaments (circle A) and thick, myosin-like filament (circle M) can be distinguished. Arrow points to thin, smooth filaments in platelet process. × 75,000.

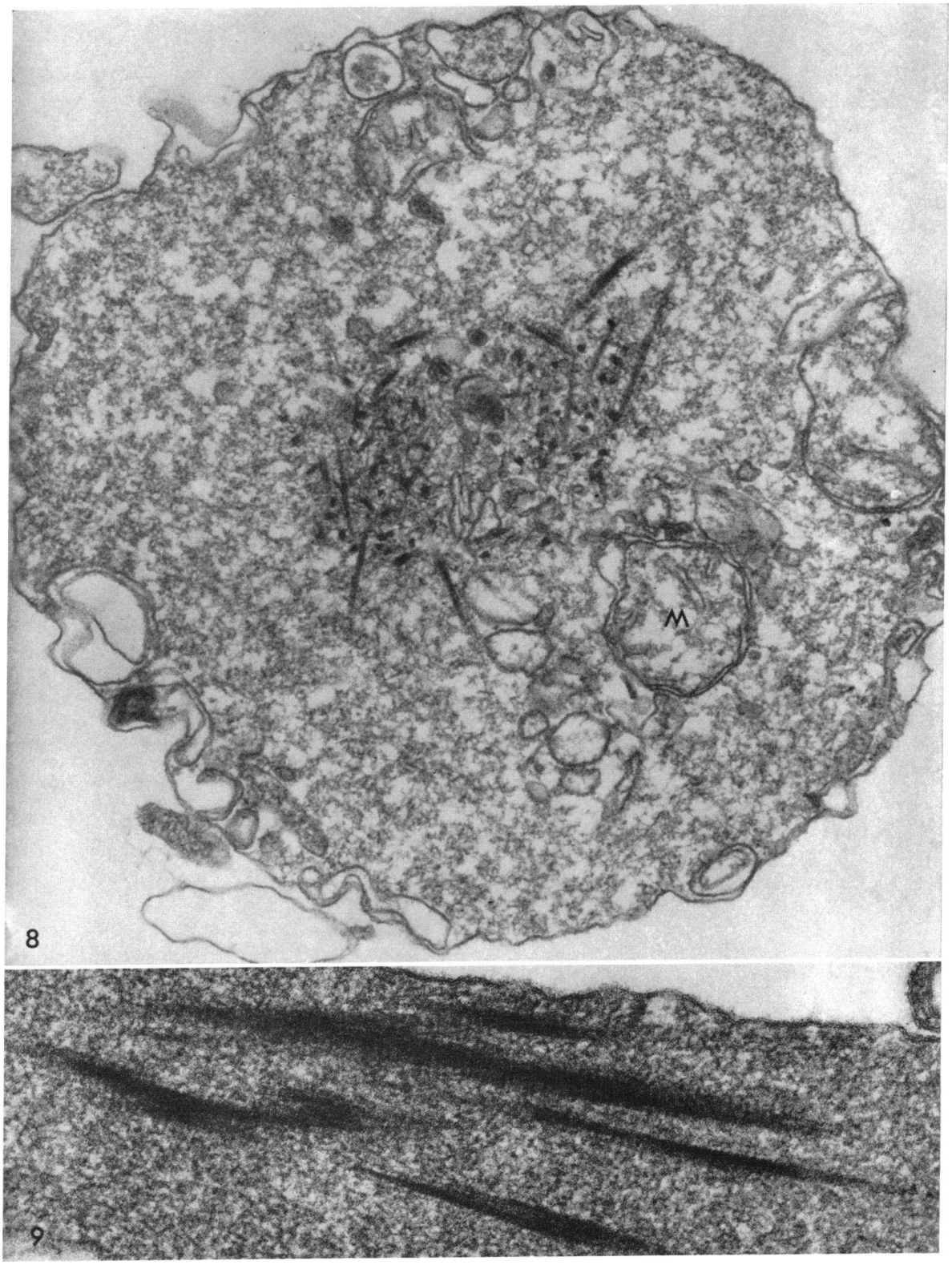


FIGURE 8 Tactoids or thick filament aggregates formed in platelet treated with ATP and $MgCl_2$. Filaments clearly taper at both ends. Diameters of cross sections depend on where along the length of the filament it has been cut. M, swollen mitochondrion. × 60,000.
FIGURE 9 Large "myosin" crystal seen in detail of platelet. Such large crystals were mostly found in platelets with dense cytoplasm. Note periodic cross striations which measured ±110 A. × 108,000.

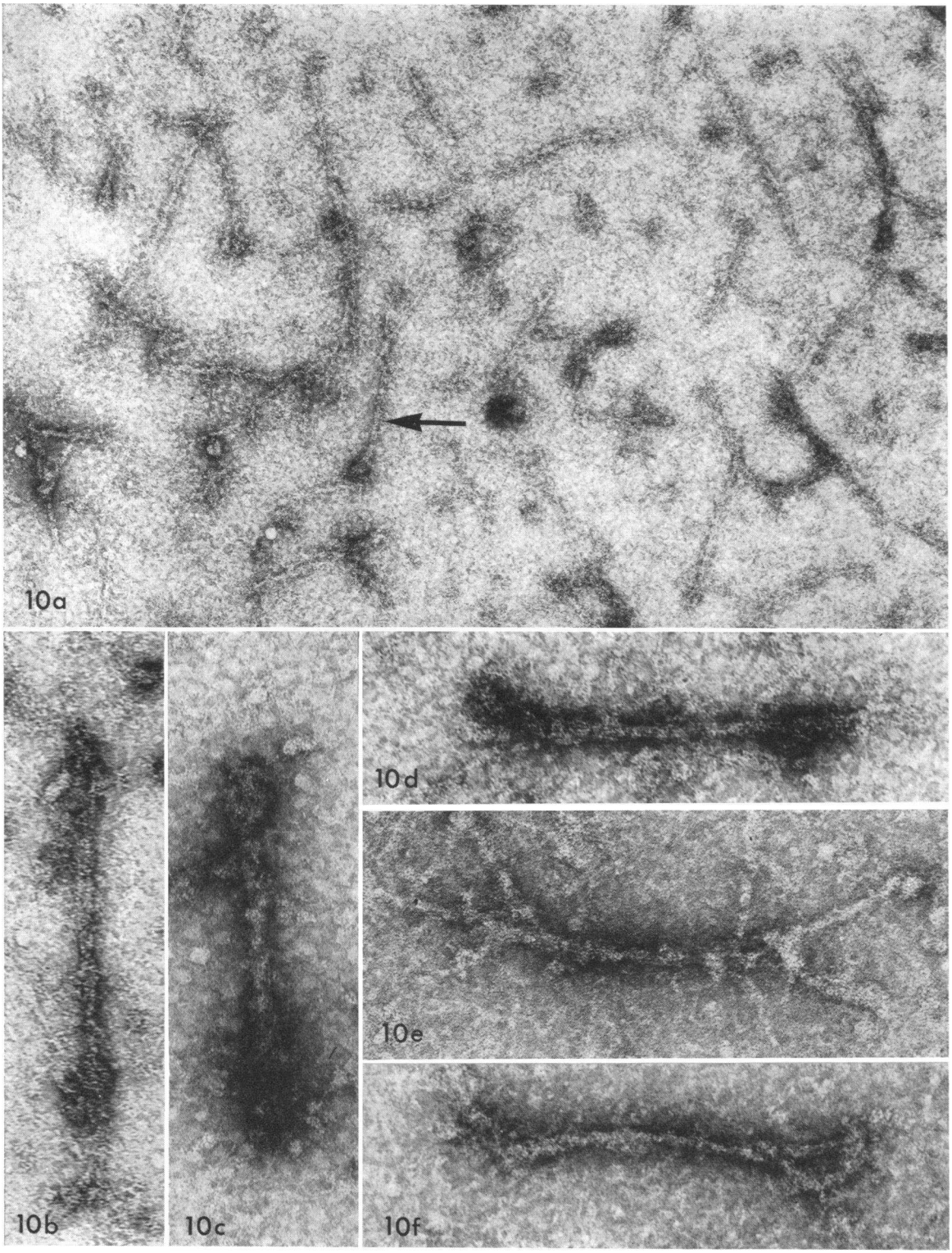


FIGURE 10 Negatively stained myosin-like filaments extracted from bovine platelets, Fig. 10a, survey at low power to show uniformity of filaments. Arrow points to a filament which terminates in a bulb on one end and has a frayed appearance on the other. × 88,000. Figs. 10b–f, variety of myosin-like filaments encountered in bovine platelet extracts. Note uniformity of total length as well as length of "smooth" shaft. Projections or knob formations are seen at the ends. × 160,000.

of isolated actomyosin (4). Moreover, all actin filaments examined to date have had the ability to form ATP-dissociable complexes with purified HMM. This observation was utilized to further characterize the filaments seen in platelets. Our observation that HMM combined with the 70–80 A filaments to form typical arrowhead complexes offers confirmatory evidence that these structures represent actin. The ability of HMM derived from rabbit myosin to react with actin from human as well as bovine platelets is interesting, but not surprising since it has been shown that this moiety of the myosin molecule can combine with purified actin of even less related organisms such as Mytilus adductor muscle (9), Acanthamoeba actin (11), actin filaments derived from Physarum (10), and Dictyostelium (12), and that derived from sea urchin eggs (31). There have been no reports that nonactin filaments can react with HMM to form ATP-dissociable complexes, and in the studies presented here HMM did not seem to combine with microtubules or disrupted membranes.

Identification of myosin filaments in well fixed embedded platelets proved to be more difficult than the demonstration of actin-like filaments. As has been the case with smooth muscle, some in vitro manipulation seems to be required for myosin molecules to aggregate into filaments resolvable by electron microscopy. It is generally known that in well fixed relaxed smooth muscle only the thin filaments are visible. However, aggregates believed to be myosin filaments have been demonstrated in guinea pig taenia coli and chicken gizzard smooth muscle when these tissues were studied in a contracted state (18). In very detailed studies on smooth muscle myosin, Shoenberg (32) suggested that Ca^{++}, Mg^{++}, and excess ATP must be present for thick filament formation, conditions which certainly prevailed in the experiments reported here. Myosin aggregates have also been visualized in trypsin-treated smooth muscle of the toad intestine (19) where they appeared as clumps of tactoids very similar in dimension to those illustrated in Figs. 6–8. Finally, treatment of platelets with trypsin appears to lead to similar results (33). To this should be added that in a very recent series of studies, it has been suggested that all of the large myosin aggregates described in the foregoing may be shrinkage artefacts (34, 35). This possibility could explain why the largest aggregates were usually found in platelets with very dense cytoplasm (Fig. 9). On the other hand, the large crystals are also reminiscent of similar structures which form by aggregation of light meromyosin (LMM) after removal of the HMM moiety from myosin by mild tryptic digestion (15). Huxley has proposed that at low ionic strengths aggregates of LMM could grow to microscopic dimensions increasing indefinitely in length as well as in width, since these rods lack HMM lateral projections (9). Fine filaments measuring 20–30 A in width resolved in synthetic LMM crystals are also seen in the platelet crystals. However, if these crystalline structures indeed consist of LMM one would have to postulate an enzymatic process by which HMM is split off the myosin molecule. It is possible that the proteolytic enzymes known to be present in platelet lysosomes (36, 37) could effect this reaction, but at the present time there are no data to substantiate this hypothesis.

The configuration and dimensions of the myosin aggregates extracted from platelets were identical to those extracted from vertebrate smooth muscle (23) and synthetically prepared myosin from striated muscle (9). Immunochemical, or histochemical analyses as well as X-ray diffraction studies are needed for full characterization of these proteins on the molecular level.

As is the case in smooth muscle, the mechanism by which these filamentous proteins interact to cause contraction is baffling. In striated muscle contraction and relaxation are associated with spatial rearrangements of existing actin and myosin filaments. On the other hand, it has been suggested that in smooth muscle, soluble myosin molecules aggregate into filaments only during contraction or under specific experimental conditions. A similar situation appears to obtain in platelets. Within the smooth muscle cell, actin filaments are believed to be anchored to the sarcolemma, and it has been proposed that during contraction the interaction of the newly formed myosin filaments with the existing actin filaments results in the deformation characteristic of the contracted state of this cell. Submembranous, actin-like filaments have also been resolved in platelets (28). If aggregation of myosin molecules would take place in contiguity with these submembranous filaments a similar interaction could be operative here.

ACKNOWLEDGMENTS

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Mr. Grusky's participation in these studies were in partial fulfillment of the requirements for a Ph.D. degree from Fordham University.

Note added in proof: During the past year related or similar observations have been made independently in several laboratories, and the following relevant publications have appeared:

Adelstein, R. S., T. D. Pollard, and W. M. Kuehl. 1971. Isolation and characterization of myosin and two myosin fragments from human blood platelets. *Proc. Nat. Acad. Sci. U. S. A.* **68**: 2703.

Behnke, O., B. I. Kristensen, and L. Engelahl Nielsen. 1971. Electron microscopial observations on actinoid and myosinoid filaments in blood platelets. *J. Ultrastruct. Res.* **37**: 351.

In addition, our attention has been called to the following work dealing primarily with thrombosthenin M polymerization:

Bettex-Galland, M., E. F. Luscher, and E. R. Weibel. 1969. Thrombosthenin. Electron microscopial studies on its localization in human blood platelets and some properties of its subunits. *Throm. Diath. Haemorrh.* **22**: 431.

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Luscher, E. F., and M. Bettex-Galland. 1971. Thrombosthenin. *In* The Circulating Platelet. S. A. Johnson, editor. Academic Press Inc., New York.

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